

JC07 Rec'd PCT/PTO 13 DEC 2001

Form PTO 1300 (REV 5-93)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER P32329
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) <b>10,018712</b>
INTERNATIONAL APPLICATION NO. PCT/GB00/02365	INTERNATIONAL FILING DATE 16 June 2000	PRIORITY DATE CLAIMED 16 June 1999	
TITLE OF INVENTION <b>POLYHYDROXY DIAMINE SURFACTANTS AND THEIR USE IN GENE TRANSFER</b>			
APPLICANT(S) FOR DO/EO/US Patrick CAMILLERI, Jan Bernard ENGBERTS, Nicolaas FREDERIK, Matthew Leigh FIELDEN, and Andreas KREMER			

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11. to 16. below concern other document(s) or information included:**

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98; and Form PTO-1449.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☒ Please amend the specification by inserting before the first line the sentence: This is a 371 of International Application PCT/GB00/02365, filed 16 June 2000, which claims benefit from the following Provisional Application: GB 9914085.7, filed 16 June 1999.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☒ An Abstract on a separate sheet of paper.
19. ☐ Other items or information:

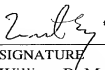
US APPLICATION NO. 10018712 (37 CFR 1.50)		INTERNATIONAL APPLICATION NO. PCT/GB00/02365		ATTORNEYS DOCKET NO. P32329	
20. [X] The following fees are submitted:				CALCULATIONS PTO USE ONLY	
Basic National Fee (37 C.F.R. 1.492(a)(1)-(5)):				<b>\$900.00</b>	
Search Report has been prepared by the EPO or JPO .....\$890.00					
International Preliminary Examination Fee paid to USPTO (37 CFR 1.492) .....\$710.00					
No International Preliminary Examination Fee paid to USPTO (37 CFR 1.492) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .....\$740.00					
Neither International Preliminary Examination Fee (37 CFR 1.492) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$1,040.00					
International Preliminary Examination Fee paid to USPTO (37 CFR 1.492) and all claims satisfied provisions of PCT Article 33(2)-(4).....\$100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				<b>\$900.00</b>	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				<b>\$0.00</b>	
Claims	Number Filed	Number Extra	Rate		
Total claims	21 - 20 =	1	1 x \$18.00	<b>\$18.00</b>	
Independent claims	2 - 3 =	0	0 x \$84.00	<b>\$0.00</b>	
Multiple dependent claims (if applicable)			+ \$280.00	<b>\$0.00</b>	
TOTAL OF ABOVE CALCULATIONS =				<b>\$908.00</b>	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				<b>\$</b>	
SUBTOTAL =				<b>\$908.00</b>	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)) +				<b>\$</b>	
TOTAL NATIONAL FEE =				<b>\$908.00</b>	
				Amount to be refunded	<b>\$</b>
				charged	<b>\$</b>

- a. ☐ A check in the amount of \$\_\_\_\_\_ to cover the above fees is enclosed.
- b. ☒ Please charge my Deposit Account No. 19-2570 in the amount of **\$908.00** to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-2570. A duplicate copy of this sheet is enclosed.
- d. ☒ General Authorization to charge any and all fees under 37 CFR 1.16 or 1.17, including petitions for extension of time relating to this application (37 CFR 1.136 (a)(3)).

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE  
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REGISTRATION NO.

PATENT

ATTORNEY'S DOCKET NUMBER P32329

TRANSMITTAL LETTER TO THE U.S. DESIGNATED OFFICE  
(DO/US) - ENTRY INTO NATIONAL STAGE UNDER 35 USC 371

INTERNATIONAL APP. NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB00/02365	16 June 2000	16 June 1999

TITLE OF INVENTION  
POLYHYDROXY DIAMINE SURFACTANTS AND THEIR USE IN GENE TRANSFER

APPLICANT(S) FOR DO/US

Patrick CAMILLERI, Jan Bernard ENGBERTS, Nicolaas FREDERIK, Matthew Leigh  
FIELDEN, and Andreas KREMER

Box PCT

Assistant Commissioner for Patents

Washington, D.C. 20231

ATTENTION: DO/US

## CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this Transmittal Letter, Form PTO 1390 and the papers indicated as being transmitted therewith, and Post Card are being deposited with the United States Postal Service on this date December 13, 2001 in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EV000522262US addressed to the:

Assistant Commissioner for Patents, Washington, D.C. 20231.

Elsa Matos

(Typed or printed name of person mailing paper)

Elsa Matos

(Signature of person mailing paper)



20462

PATENT TRADEMARK OFFICE

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Date of Deposit: December 13, 2001

531 Rec'd PC

13 DEC 2001

**Attorney Docket No: P32329**

**IN THE UNITED STATES INTERNATIONAL EXAMINING AUTHORITY**

International Application No.: PCT/GB00/02365  
International Filing Date: 16 June 2000  
Priority Date Claimed: 16 June 1999  
Applicants for DO/US: Camilleri, et al.  
Title of Invention: Polyhydroxy Diamine Surfactants and Their Use in Gene Transfer

Assistant Commissioner for Patents  
Box PCT  
Washington D.C. 20231

**FIRST PRELIMINARY AMENDMENT**

Sir:

Preliminary to calculating filing fees and examining this application please amend the application as follows.

**In the Specification:**

Please add the following paragraph to page 1, directly under the Title of the Invention with the following paragraph:

-- CROSS REFERENCES TO RELATED APPLICATIONS--

This application is a National Stage Application filed under 35 U.S.C. §371 of PCT/GB00/02365, filed on June 16, 2000--.

After page 14, please insert the Abstract that accompanies this Preliminary Amendment.

Int'l. App. No.: PCT/GB00/02365

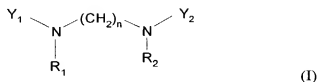
Int'l. Filing Date: 16 June 2000

**In the Claims:**

Please cancel claims 1-20.

Please add new claims 21-41.

21. (Amended) A method of transferring a DNA or RNA polynucleotide or analog thereof into a eukaryotic or prokaryotic cell *in vivo* or *in vitro*, the method comprising contacting the cell with a DNA or RNA polynucleotide or analog thereof and a compound of formula (I):



wherein  $Y_1$  and  $Y_2$ , which may be the same or different, are carbohydrate groups;

$R_1$  and  $R_2$ , which may be the same or different, are selected from the group consisting of:

hydrogen,

$C_{(1-24)}$  alkyl group,

$C_{(1-24)}$  alkyl carboxy group, and

a carbon chain of 2 to 24 carbon atoms having one or more carbon/carbon double bonds;

and  $n$  is from 1 to 10;

or a pharmaceutically acceptable salt thereof.

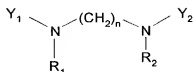
22. The method of claim 21 wherein the carbohydrate groups  $Y_1$  and  $Y_2$  are sugars.

23. The method of claim 21 wherein  $R_1$  and  $R_2$  are alkyl groups of chain-length  $C_{(10-20)}$  and  $n$  is between 2 and 8.

24. The method of claim 23 wherein  $R_1$  and  $R_2$  are alkyl groups of chain-length  $C_{(12-18)}$  and  $n$  is 4 or 6.

25. The method of claim 21 wherein  $R_1$  and  $R_2$  are carbon chains of 2 to 24 carbon atoms having one or more carbon/carbon double bonds.

26. The method of claim 25 wherein the carbon chains have 18 carbon atoms.
27. The method of claim 21 wherein the compound is symmetrical, that is the groups  $R_1$  and  $R_2$  are the same, and  $Y_1$  and  $Y_2$  are the same.
28. The method of claim 21 wherein the polynucleotide is transferred into the cell to achieve an antisense knock-out effect.
29. The method of claim 21 wherein the polynucleotide is transferred into the cell for gene therapy.
30. The method of claim 21 wherein the polynucleotide is transferred into the cell for genetic immunization (for the generation of antibodies) in whole organisms.
31. The method of claim 21 wherein the polynucleotide is transferred into the cell in culture.
32. A compound of formula (I):

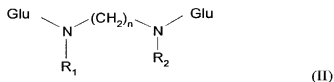


(I)

wherein  $Y_1$  and  $Y_2$ , which may be the same or different, are carbohydrate groups;  $R_1$  and  $R_2$ , which may be the same or different, are selected from the group consisting of hydrogen,  $C_{(1-24)}$  alkyl group,  $C_{(1-24)}$  alkylcarboxy group, and a carbon chain of 2 to 24 carbon atoms having one or more carbon/carbon double bonds; and  $n$  is from 1 to 10; or a pharmaceutically acceptable salt thereof.

33. The compound of claim 32 wherein  $R_1$  and  $R_2$  are alkyl groups of chain-length  $C_{(10-20)}$  and  $n$  is between 2 and 8.

34. The compound of claim 33 wherein  $R_1$  and  $R_2$  are alkyl groups of chain-length  $C_{(12-18)}$  and  $n$  is 4 or 6.
35. The compound of claim 32 wherein the compound is a gemini compound where  $R_1$  and  $R_2$  are the same and  $Y_1$  and  $Y_2$  are the same.
36. The compound of claim 35 which has the formula (II):



wherein Glu is glucose in open chain form (glucitol).

37. The compound of claim 32 wherein one of  $R_1$  or  $R_2$  is an alkyl group of chain-length  $C_{(1-24)}$ , and the other is a  $C_{(1-24)}$  alkyl carboxy group.
38. The compound of claim 32 wherein  $R_1$  and  $R_2$  are carbon chains of 2 to 24 carbon atoms having one or more carbon/carbon double bonds.
39. The compound of claim 38 wherein the carbon chain has 18 carbon atoms.
40. A method of transferring a polynucleotide or an anti-infective compound into a prokaryotic or eukaryotic organism for use in anti-infective therapy, the method comprising contacting the organism with the compound of claim 32 and a polynucleotide or anti-infective compound.
41. A process for preparing the compound of claim 32 comprising the addition of carbohydrate groups at the amine ends of an alkyl diamine compound.

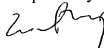
Int'l App. No.: PCT/GB00/02365

Int'l. Filing Date: 16 June 2000

### REMARKS

This Preliminary Amendment is being made upon entry of International Application No. PCT/GB00/02365 into the U.S. National Phase of prosecution. Claim 1-20 have been cancelled. Claims 21-41 have been added to eliminate multiple dependencies and to comply with proper U.S. claim format. Furthermore, attached hereto is a marked-up version of the changes made to the application by the current preliminary amendment. The attached page is captioned, "**Version with markings to show changes made.**"

Respectfully submitted,



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### New Use

This invention relates to new uses for carbohydrate-based surfactant compounds.

- 5 Such uses include facilitating the transfer of compounds into cells for drug delivery and facilitating the transfer of oligonucleotides and polynucleotides into cells for gene expression studies or gene therapy. The invention also relates to new carbohydrate-based surfactant compounds and methods for their production.

- 10 Surfactants are substances that markedly affect the surface properties of a liquid, even at low concentrations. For example surfactants will significantly reduce surface tension when dissolved in water or aqueous solutions and will reduce interfacial tension between two liquids or a liquid and a solid. This property of surfactant molecules has been widely exploited in industry, particularly in the detergent and oil industries. In the 1970s a new class of surfactant molecule was reported, characterised by two hydrophobic chains
- 15 with polar heads which are linked by a hydrophobic bridge (Deinega, Y *et al.*, *Kolloidn. Zh.* 36, 649, 1974). These molecules, which have been termed "gemini" (Menger, FM and Littau, CA, *J. Am. Chem. Soc.* 113, 1451, 1991), have very desirable properties over their monomeric equivalents. For example they are highly effective in reducing interfacial tension between oil and water based liquids and have a very low critical micelle
- 20 concentration. Recently, Pestman *et al* have reported the synthesis and characterisation of nonionic carbohydrate-based gemini surfactants (Pestman, JM *et al*, *Langmuir*, 13, 6857-6860, 1997).

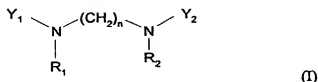
- Cationic surfactants have been used *inter alia* for the transfection of polynucleotides into cells in culture, and there are examples of such agents available
- 25 commercially to scientists involved in genetic technologies (for example the reagent TEx<sup>TM</sup>-50 for the transfection of eukaryotic cells available from Promega Corp. WI, USA).

- The efficient delivery of DNA to cells *in vivo*, either for gene therapy or for antisense therapy, has been a major goal for some years. Much attention has concentrated on the use of viruses as delivery vehicles, for example adenoviruses for epithelial cells in
- 30 the respiratory tract with a view to corrective gene therapy for cystic fibrosis (CF). However, despite some evidence of successful gene transfer in CF patients, the adenovirus route remains problematic due to inflammatory side-effects and limited transient expression

of the transferred gene. Several alternative methods for *in vivo* gene delivery have been investigated, including studies using cationic surfactants. Gao, X *et al.* (1995) *Gene Ther.* 2, 710-722 demonstrated the feasibility of this approach with a normal human gene for CF transmembrane conductance regulator (CFTR) into the respiratory epithelium of CF mice using amine carrying cationic lipids. This group followed up with a liposomal CF gene therapy trial which, although only partially successful, demonstrated the potential for this approach in humans (Caplen, NJ. *et al.*, *Nature Medicine*, 1, 39-46, 1995). More recently other groups have investigated the potential of other cationic lipids for gene delivery, for example cholesterol derivatives (Oudrhiri, N *et al.* *Proc. Natl. Acad. Sci.* 94, 1651-1656, 1997). This limited study demonstrated the ability of these cholesterol based compounds to facilitate the transfer of genes into epithelial cells both *in vitro* and *in vivo*, thereby lending support to the validity of this general approach.

These studies, and others, show that in this new field of research there is a continuing need to develop novel low-toxicity surfactant molecules to facilitate the effective transfer of polynucleotides into cells both *in vitro* for transfection in cell-based experimentation and *in vivo* for gene therapy and antisense treatments. The present invention seeks to overcome the difficulties exhibited by existing compounds.

The invention relates to the use of carbohydrate-based surfactant compounds having the general formula (I):

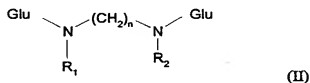


wherein  $Y_1$  and  $Y_2$ , which may be the same or different, are carbohydrate groups, preferably sugars;

$R_1$  and  $R_2$ , which may be the same or different, are selected from:

- a) hydrogen;
- b)  $C_{(1-24)}$  alkyl group;
- c)  $C_{(1-24)}$  alkyl carboxy group; or

- d) a carbon chain of 2 to 24 carbon atoms having one or more carbon/carbon double bonds,  
and n is from 1 to 10;  
or a salt, preferably a pharmaceutically acceptable salt thereof,
- 5 for facilitating the transfer of DNA or RNA polynucleotides, or analogs thereof, into a eukaryotic or prokaryotic cell *in vivo* or *in vitro*.
- Preferably the compound is symmetrical, that is the groups  $R_1$  and  $R_2$  are the same, and  $Y_1$  and  $Y_2$  are the same. The molecular symmetry allows these compounds to be referred to as "gemini" surfactants.
- 10 In a preferred embodiment, the carbohydrate groups  $Y_1$  and  $Y_2$  are sugars, attached to the nitrogen via a reduced imine bond. Such sugars include monosaccharides such as glucose and fructose, disaccharides such as lactose and more complex sugars, for instance sugars based on cellulose.
- In a particularly preferred embodiment,  $Y_1$  and  $Y_2$  are glucose; the compounds
- 15 having the general structure of formula (II):

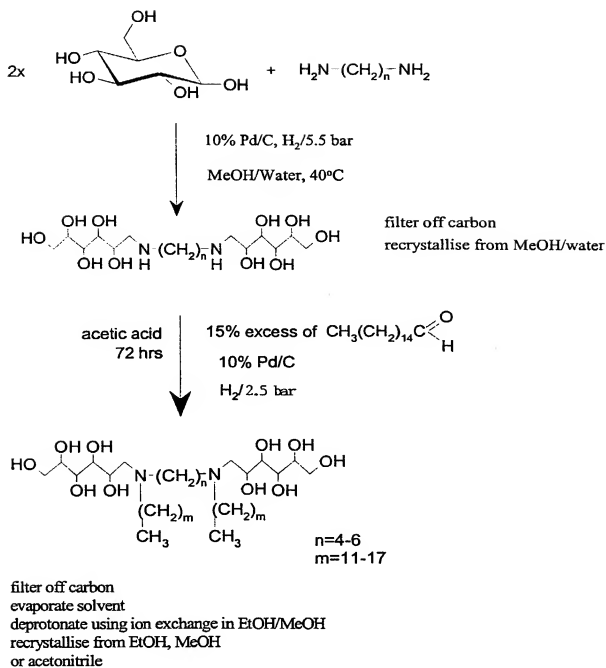


- wherein Glu is glucose in open chain form (glucitol) linked via the C-1 (aldehyde carbon),  
and  $R_1$ ,  $R_2$  and n are as hereinbefore defined.
- 20 In a further preferred embodiment  $R_1$  and  $R_2$  are alkyl groups of chain-length  $C_{(10-20)}$ , most preferably  $C_{(12-18)}$ , and n is between 2 and 8, most preferably 4 or 6.
- In a still further preferred embodiment  $R_1$  and  $R_2$  are  $C_{(12-24)}$ , preferably  $C_{(16-20)}$ , most preferably  $C_{18}$  carbon chains having one or more carbon/carbon double bonds.
- 25 Such compounds are new and form part of the present invention.
- The present invention shows the surprising finding that carbohydrate-based surfactants are highly efficient agents for facilitating the transfection of polynucleotides into cells.

Compounds of formula (I) in which  $R_1$  and  $R_2$  are not both  $C_{(1-24)}$  alkyl carboxyl groups are new. Accordingly, in a further aspect, the present invention provides for compounds of formula (I) in which one of  $R_1$  or  $R_2$  is an alkyl group of chain-length  $C_{(1-24)}$ , and the other is a  $C_{(1-24)}$  alkyl carboxy group.

- 5       Compounds of the present invention may be prepared from readily available starting materials using synthetic chemistry well known to the skilled person. A general process for preparing carbohydrate-based surfactant compounds comprises the addition of carbohydrate groups at the amine ends of an alkyl diamine compound. The following is a general scheme (scheme 1) for the synthesis of the sugar-based compounds of the
- 10   invention, as illustrated for glucose-based compounds:

Scheme 1

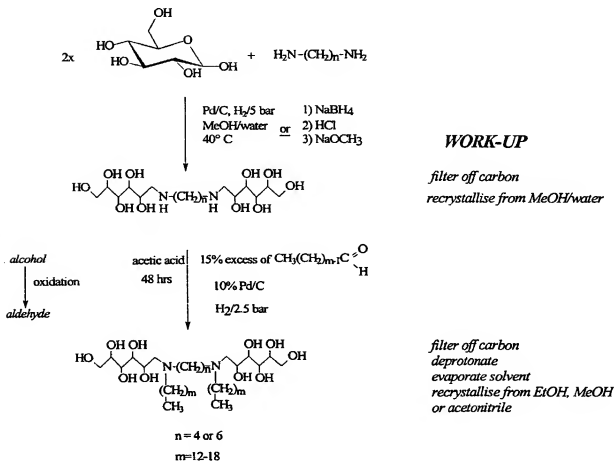


For  $R_1 = C_{(1-24)}$  alkyl carboxy, the second step will be the formation of an amide bond, using a suitable acylating agent, for instance an activated derivative of the corresponding acid.

Preferably the scheme for the synthesis of the sugar-based compounds of the invention, as illustrated for glucose-based compounds, is as shown in scheme 2:

Scheme 2

5



In a further aspect, the compounds of the invention which comprise carbon chains of 2 to 24 carbon atoms and having one or more carbon/carbon double bonds may be prepared according to scheme 3 (figure 3) as exemplified for the C<sub>18</sub> oleyl compound. The skilled person can use this information to devise analogous processes for preparing other compounds comprising carbon chains of 2 to 24 carbon atoms and having one or more carbon/carbon double bonds.

The processes described above are for the synthesis of symmetrical, that is "gemini", carbohydrate-based surfactants. Non-symmetrical carbohydrate-based

surfactants of the invention can be prepared by introducing asymmetry, for example at the primary amines of the diamine, by using different protecting groups.

- In a further aspect, the carbohydrate-based surfactant compounds are used to facilitate the transfer of oligonucleotides and polynucleotides into cells to achieve an antisense knock-out effect, for gene therapy and genetic immunisation (for the generation of antibodies) in whole organisms. In a further preferred embodiment, the carbohydrate-based surfactant compounds are used to facilitate the transfection of polynucleotides into cells in culture when such transfer is required, in, for example, gene expression studies and antisense control experiments among others. The polynucleotides can be mixed with the compounds, added to the cells and incubated to allow polynucleotide uptake. After further incubation the cells can be assayed for the phenotypic trait afforded by the transfected DNA, or the levels of mRNA expressed from said DNA can be determined by Northern blotting or by using PCR-based quantitation methods for example the Taqman® method (Perkin Elmer, Connecticut, USA). Compounds of the invention offer a significant improvement, typically between 3 and 6 fold, in the efficiency of cellular uptake of DNA in cells in culture, compared with compounds in the previous art. In the transfection protocol, the gemini compound may be used in combination with one or more supplements to increase the efficiency of transfection. Such supplements may be selected from, for example:
- (i) a neutral carrier, for example dioleoyl phosphatidylethanolamine (DOPE) (Farhood, H., *et al* (1985) *Biochim. Biophys. Acta* 1235 289);
  - (ii) a complexing reagent, for example the commercially available PLUS reagent (Life Technologies Inc. Maryland, USA) or peptides, such as polylysine or polyornithine peptides or peptides comprising primarily, but not exclusively, basic amino acids such as lysine, ornithine and/or arginine. The list above is not intended to be exhaustive and other supplements that increase the efficiency of transfection are taken to fall within the scope of the invention.

In still another aspect, the invention relates to the transfer of genetic material in gene therapy using the compounds of the invention.

- Yet another aspect of the invention relates to methods to effect the delivery of non-nucleotide based drug compounds into cells *in vitro* and *in vivo* using the compounds of the invention.

In a further aspect, the invention relates to methods to facilitate the transfer of a polynucleotide or an anti-infective compounds into prokaryotic or eukaryotic organism for use in anti-infective therapy.

The following definitions are provided to facilitate understanding of certain  
5 terms used frequently herein.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-  
10 stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one  
15 or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications have been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA  
20 and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Transfection" refers to the introduction of polynucleotides into cells in culture using methods involving the modification of the cell membrane either by chemical or physical means. Such methods are described in, for example, Sambrook et al.,  
25 *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). The polynucleotides may be linear or circular, single-stranded or double-stranded and may include elements controlling replication of the polynucleotide or expression of homologous or heterologous genes which may comprise part of the polynucleotide.

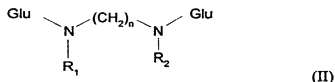
30

The invention will now be described by way of the following examples.



**Example 1 – Transfection of recombinant plasmid expressing luciferase into cells in culture using carbohydrate-based surfactant compounds.**

Carbohydrate-based surfactant compounds having the general structure of formula (II)



- 5 were synthesised according to the method as hereinbefore described. The following compounds were made:

Compound no. R<sub>1</sub>-n-R<sub>2</sub>

	GS_G_1:	16-6-16
10	GS_G_2:	18-6-18 (unsaturated (oleyl) R chains)
	GS_G_3:	12-6-12
	GS_G_4:	14-6-14
	GS_G_5:	14-4-14
	GS_G_6:	16-4-16
15	GS_G_7:	12-4-12
	GS_G_8:	18-4-18
	GS_G_9:	18-6-18

- 20 Transfection studies were performed using the adherent cell line CHO-K1 (Puck et al. 1958). Complete medium consisted of MEM alpha medium supplemented with 10 % v/v foetal bovine serum and 1x L-Glutamine. All media and supplements were obtained from Life Technologies.

- 25 Stable transfected cell lines expressing β-galactosidase were generated by cotransfection of the plasmid pSV-β-Galactosidase Control Vector (Promega) with the plasmid Selecta Vecta-Neo (R & D Systems) in a 10:1 ratio. Following G418 (Life Technologies) selection (0.8 mg ml<sup>-1</sup>), candidate cell lines were tested for β-galactosidase activity (β-Gal Reporter Gene Assay, chemiluminescent; Roche Diagnostics).

***In Vitro* Gene Transfection.**

Cells were seeded into 96-well plates (Beckton Dickinson) 16-18 hours prior to transfection at an approximate density of  $1 \times 10^4$  cells per well. For transfection, 64 ng of the luciferase reporter gene plasmid, pGL3-Control Vector (Promega) per well, was incubated with various concentrations of the carbohydrate-based gemini compounds.

- 5 After 30 minutes incubation at RT, OPTI-MEM® medium (Life Technologies) was added to the transfection mixture and the solution placed on the cells (final volume per well: 100 µl). Following a 3 hour or over night incubation at 37°C, the transfection solution was replaced with complete medium and the cells incubated further at 37°C. Reporter gene assays were performed according to the manufacturer's guidelines (Roche
- 10 Diagnostics) approximately 48 hours post transfection. Luminescence was measured in a Packard TopCount NXT Microplate Scintillation and Luminescence Counter. For normalization purpose, β-galactosidase activity (β-Gal Reporter Gene Assay, chemiluminescent; Roche Diagnostics) was measured and luciferase activity per β-galactosidase activity was calculated. The results are shown in Figures 1 and 2.

15

**Example 2 – Transfection efficiency of GS\_G\_2 in the presence or absence of foetal calf serum (FCS)**

GS\_G\_2 was prepared as described hereinabove and used in experiments to test the transfection efficiency of the compound as described in example 1. Two experiments

20 were conducted, in both experiments the compound was tested at 4uM, 8uM, 10uM, 20uM and 30uM both in the presence and absence of PLUS reagent. In the first experiment the CHO-K1 cells were incubated overnight without foetal calf serum (FCS) and in the second experiment the CHO-K1 cells were incubated overnight in the presence of FCS. The results showed that preincubation with foetal calf serum had no

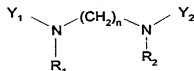
25 effect on the transfection efficiency of the GS\_G\_2 compound. This result was surprising as it is well known in the art that serum reduces transfection efficiency. The presence or absence of PLUS reagent had no significant effect on transfection efficiency in either experiment.

**Brief description of the drawings.**

- 5 Fig 1. Transfection of CHO-K1 cells (stable transfected with beta-galactosidase) with carbohydrate-based gemini compounds GS-G-3, GS-G-4, GS-G-5, GS-G-6, GS-G-7, GS-G-8, and GS-G-9. Concentrations of the compounds in  $\mu\text{M}$  is shown on the x axis. Bars represent the mean cps (counts per second) of 8 experiments  $\pm$  the standard error of the mean.
- 10 Fig 2. Transfection of CHO-K1 cells (stable transfected with beta-galactosidase) with carbohydrate-based gemini compound GS-G-1. Concentrations of the compound in  $\mu\text{M}$  is shown on the x axis. Bars represent the mean cps (counts per second) of 8 experiments  $\pm$  the standard error of the mean.
- 15 Fig 3. Scheme 3 shows a general process for the preparation of an oleyl compound of the invention.

## CLAIMS

1. The use of carbohydrate-based surfactant compounds having the general formula (I):



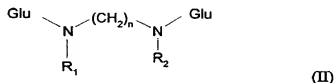
(I)

wherein  $Y_1$  and  $Y_2$ , which may be the same or different, are carbohydrate groups;  
 $R_1$  and  $R_2$ , which may be the same or different, are selected from:

- a) hydrogen;
  - b)  $C_{(1-24)}$  alkyl group;
  - c)  $C_{(1-24)}$  alkyl carboxy group; or
  - d) a carbon chain of 2 to 24 carbon atoms having one or more carbon/carbon double bonds,
- and  $n$  is from 1 to 10;
- or a salt, preferably a pharmaceutically acceptable salt thereof,
  - for facilitating the transfer of DNA or RNA polynucleotides, or analogs thereof, into a eukaryotic or prokaryotic cell *in vivo* or *in vitro*.
2. The use according to claim 1 wherein the carbohydrate groups  $Y_1$  and  $Y_2$  are sugars.
3. The use according to claim 1 or 2 wherein  $R_1$  and  $R_2$  are alkyl groups of chain-length  $C_{(10-20)}$  and  $n$  is between 2 and 8.
4. The use according to claim 3 wherein  $R_1$  and  $R_2$  are alkyl groups of chain-length  $C_{(12-18)}$  and  $n$  is 4 or 6.

5. The use according to claim 1 wherein  $R_1$  and  $R_2$  are carbon chains of 2 to 24 carbon atoms having one or more carbon/carbon double bonds.
6. The use according to claim 5 wherein the carbon chains have 18 carbon atoms.
7. The use according to any one of claims 1 to 6 wherein the carbohydrate-based surfactant compound is symmetrical, that is the groups  $R_1$  and  $R_2$  are the same, and  $Y_1$  and  $Y_2$  are the same.
8. The use according to any one of claims 1 to 7 wherein the oligonucleotides or polynucleotides are transferred into cells to achieve an antisense knock-out effect.
9. The use according to any one of claims 1 to 7 wherein the oligonucleotides or polynucleotides are transferred into cells for gene therapy.
10. The use according to any one of claims 1 to 7 wherein the oligonucleotides or polynucleotides are transferred into cells for genetic immunisation (for the generation of antibodies) in whole organisms.
11. The use according to any one of claims 1 to 7 wherein the oligonucleotides or polynucleotides are transferred into cells in culture.
12. A carbohydrate-based surfactant compound as defined in claim 1 wherein  $R_1$  and  $R_2$  are alkyl groups of chain-length  $C_{(10-20)}$  and  $n$  is between 2 and 8.
13. A carbohydrate-based surfactant compound according to claim 12 wherein  $R_1$  and  $R_2$  are alkyl groups of chain-length  $C_{(12-18)}$  and  $n$  is 4 or 6.
14. A carbohydrate-based surfactant compound according to claim 12 or 13 wherein the carbohydrate-based surfactant compound is a gemini compound where  $R_1$  and  $R_2$  are the same and  $Y_1$  and  $Y_2$  are the same.

15. A carbohydrate-based surfactant compound according to claim 14 which has the formula (II):



5

wherein Glu is glucose in open chain form (glucitol).

16. A carbohydrate-based surfactant compound of formula (I) in which one of R<sub>1</sub> or R<sub>2</sub> is an alkyl group of chain-length C<sub>(1-24)</sub>, and the other is a C<sub>(1-24)</sub> alkyl carboxy group.

10

17. A carbohydrate-based surfactant compound of formula (I) in which R<sub>1</sub> and R<sub>2</sub> are carbon chains of 2 to 24 carbon atoms having one or more carbon/carbon double bonds.

15

18. A carbohydrate-based surfactant compound according to claim 17 wherein the carbon chain has 18 carbon atoms.

19. The use of a carbohydrate-based surfactant compound as defined in any one of claims 12 to 18 to facilitate the transfer of a polynucleotide or an anti-infective compound into a prokaryotic or eukaryotic organism for use in anti-infective therapy.

20

20. A process for preparing the carbohydrate-based surfactant compound of claim 12 comprising the addition of carbohydrate groups at the amine ends of an alkyl diamine compound.

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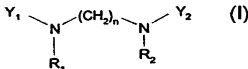
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**(54) Title:** POLYHYDROXY DIAMINE SURFACTANTS AND THEIR USE IN GENE TRANSFER

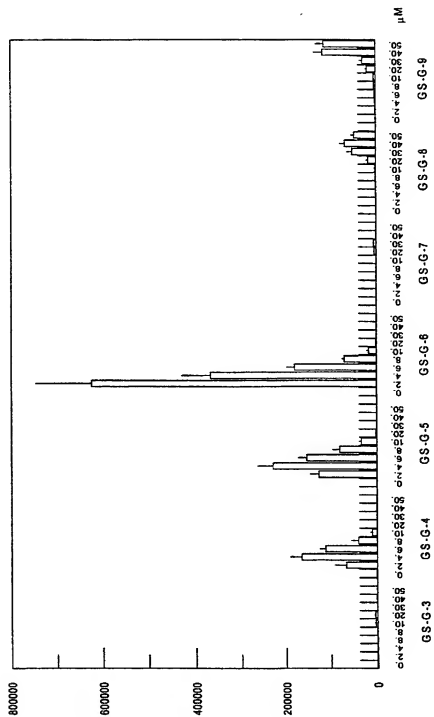


(57) **Abstract:** The use of carbohydrate-based surfactant compounds having general formula (I) wherein  $Y_1$  and  $Y_2$ , which may be the same or different, are carbohydrate groups;  $R_1$  and  $R_2$ , which may be the same or different, are selected from: a) hydrogen; b)  $C_{1-24}$  alkyl group; c)  $C_{1-24}$  alkyl carboxy group; or d) a carbon chain of 2 to 24 carbon atoms having one or more carbon/carbon double bonds, and  $n$  is from 1 to 10; for facilitating the transfer of DNA or RNA polynucleotides, or analogs thereof, into an eukaryotic or prokaryotic cell *in vivo* or *in vitro*. New carbohydrate-based surfactant compounds are also disclosed.

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Figure 1





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Figure 2

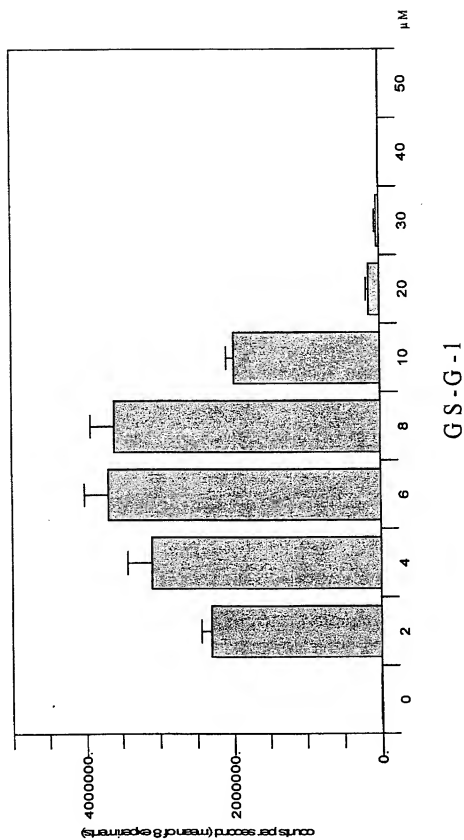
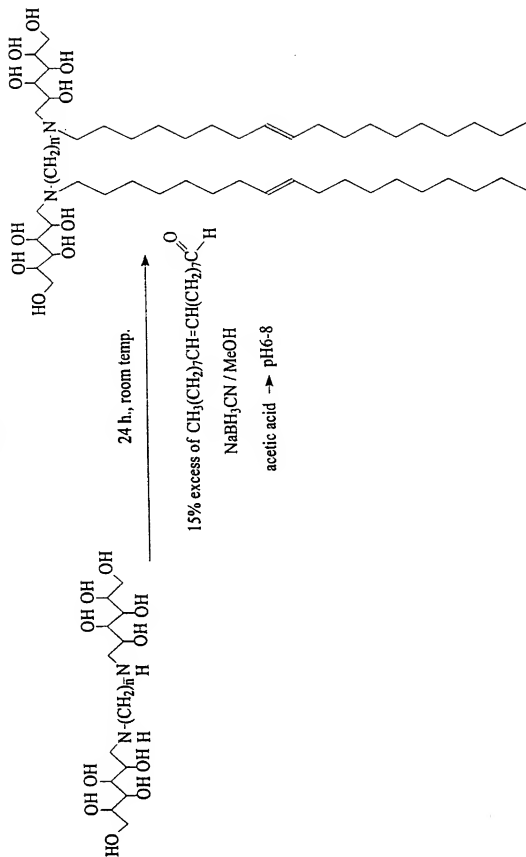


Figure 3



Docket No.: P32329

PCT/GB00/02365

## DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

## POLYHYDROXY DIAMINE SURFACTANTS AND THEIR USE IN GENE TRANSFER

the specification of which (check one)

☐ is attached hereto.

☒ was filed on 16 June 2000 as Serial No. PCT/GB00/02365  
and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or Inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

## Prior Foreign Application(s)

Number	Country	Filing Date	Priority Claimed
9914085.7	GREAT BRITAIN	16 June 1999	Yes

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

Application Number	Filing Date
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I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Serial No.	Filing Date	Status
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I hereby appoint the practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

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